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An origin of transfer of the Vwa plasmid of Yersinia pestis

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AN ORIGIN OF TRANSFER OF THE VWA PLASMID OF YERSINIA PESTIS

BY

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DISSERTATION

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Abstract

CHARACTERIZATION OF THE VWA PLASMID OF YERSINIA PESTIS

by

John R. Allen

University of New Hampshire, December, 1988

The Vwa plasmid (pYV019::Tn5) of Yersinia pestis was transduced from Escherichia coli LE392 to several F-containing strains of E. coli. Agarose gel electrophoresis of plasmid extracts prepared from the transductants revealed that both plasmids coexisted in these cells, at least to some degree, as autonomous cytoplasmic elements. Transductants demonstrably sensitive to the male specific phage MS-2 and thus carrying the F plasmid were used as donors in matings with several F⁻strains. The results of these matings indicated that the Vwa plasmid was mobilized and cotransferred at a low frequency (0.001%) along with F. Agarose gel electrophoresis of plasmid extracts prepared from the transconjugants demonstrated the presence of both plasmids. Additional matings indicated that mobilization was neither a donor nor a recipient phenomenon and that mobilization was independent of recA function, making cointegrate formation of homologous regions of the plasmids unlikely. The frequency of pYV019::Tn5 transfer to recipient cells was 100-1000 times greater than

can be accounted for by transposon-mediated cointegrate formation alone. It appeared likely that the Vwa plasmid of Yersinia pestis contained its own origin of transfer.

The non-mobilizable cloning vector pBR328 was used to create a BamH1 library of the Vwa plasmid. This library was used to clone an origin of transfer of the Vwa by selecting for those clones which mobilized pBR328 in the presence of a second conjugative plasmid (F). The origin of transfer of the Vwa plasmid was found to be contained in the BamH1-5 fragment. This fragment resulted in a ten fold increase in mobilization of pBR328 when compared to transposon-mediated (Tn 1000) cointegrate formation.

Hybridization studies between the Vwa plasmid and known conjugative plasmids from various incompatibility groups revealed that the Vwa plasmid shared sequence homology only to the Sma1-4 fragment of RP4. These results suggest the Vwa plasmid does not share extensive sequence homology with most plasmids of known incompatibility groups.

I. LITERATURE REVIEW

Taxonomy and Physiology of Yersinia pestis

Yersinia pestis, the etiologic agent of bubonic plague, is a non-motile, gram negative rod, 0.5 to 0.8 um by 1.5 to 2.0 um which exhibits bipolar staining with polychrome stains such as Waysons stain (Butler et al, 1977). Although first placed in the genus Pasteurella, this was later changed to Yersinia (van Loghem, 1945) in 1970. The genus Yersinia contains three medically important species Yersinia pestis, Yersinia pseudotuberculosis, and Yersinia entercolitica. Thal (1954) first suggested that the genus Yersinia be included amongst the Family Enterobacteriaceae because common phage sensitivities indicated a taxonomic relationship with the enteric bacteria (Girard, 1943). There is a 22% DNA-sequence homology between DNA of Esherichia coli and Yersinia pestis (Ritter and Gerloff, 1966) and the guanine/cystosine content of the latter is 45.6% (Lawton et al, 1968). Strains of Y. pestis are of three varieties; orientalis, antiqua, and mediaevalis.

Y. pestis can grow at temperatures between -2^{o}C and 45^{o}C with fastest growth at 28^{o}C (Sokhey and Habbu, 1943a). Optimal pH is between 7.2 and 7.6 and extremes of 5.0 to 9.6 are tolerated (Sokhey and Habbu, 1943b). Logarithmic growth in enriched media is characterized by generation times of 2 h or greater with final yields of up to 10^{11} bacteria per ml (Higuchi et al, 1957).

Y. pestis grown at 26°C shows a requirement for L-methionine and L-phenylalanine, and growth is further enhanced by the addition of L-isoleucine, L-valine, and glycine. For growth at 37°C, Y. pestis requires exogenous biotin, panthothenate, thiamine, and glutamic acid (Brownlow and Wessman, 1960). Growth of virulent strains of Y. pestis at 37°C in the presence of magnesium requires the addition of calcium (Higuchi et al, 1959). This trait will be discussed later with regard to the Vwa plasmid. Y. pestis contains a functional Embden-Meyerhoff pathway (Santer and Ajl, 1955) but no glucose-6-phosphate dehydrogenase, indicating the absence of the hexose monophosphate pathway. Y. pestis is a facultative anaerobe and the products of fermentation are lactate, ethanol, acetate, and formate (Englesberg et al, 1954). Y. pestis also exhibits a functional tricarboxylic acid cycle when grown with aeration (Santer and Ajl, 1954) with the oxidation of glucose, ribose, and lactate being constitutive. The ability to synthesize purines de novo is a virulence determinant and will be discussed later.

Virulence Factors

There are five established virulence factors for Y. pestis (Brubaker, 1972). Three of these factors, the ability to synthesize purines (Pur^+), to produce capsular antigen (Fra^+) and to store exogenous hemin (Pgm^+), are encoded for by chromosomal genes (Brubaker, 1984). Two of the factors are encoded in plasmids. The synthesis of pesticin (Pst^+) is associated with a 6 megadalton (Mdal) plasmid, and the production of V and W antigens (Vwa^+) with a 47 Mdal (Vwa) plasmid. The 47 Mdal plasmid is also responsible for

the dependence on calcium for growth of the bacterium at 37°C (Lcr^+). (Ben-Gurion and Shaffermann 1981; Ferber and Brubaker 1981; Portnoy and Falkow 1981). Characterization of the virulence determinants has relied on avirulent mutants which show a decrease in virulence expressed as a rise in the LD_{50}.

Purine dependence. Auxotrophic mutants of Y. pestis which require purines show a reduced virulence (Burrows and Bacon, 1958). Purine dependence in Y. pestis which is the result of a block in de novo synthesis of inosine monophosphate (IMP) shows a slight reduction in virulence in contrast to those adenine and guanine mutants blocked after IMP synthesis which show an LD_{50} of $>10^8$ cells (Brubaker, 1970).

Fraction I antigen. Fraction I antigen is a glycoprotein with a molecular weight of 20,000 to 500,000 daltons located in the capsular envelope and is produced when the organism is grown at 37°C but not at temperatures below 27°C (Fox and Higuchi,1958). This glycoprotein allows the organism to resist phagocytosis by neutrophils and monocytes. The antiphagocytic action of Fraction I antigen may be due to interference with the heat-labile opsonins of serum, particularly the complement components C4 and C2 (Williams et al, 1972). This anticomplementary property of Fraction I antigen may be a requirement for the surge of bacterial multiplication found in fatal plague infections.

Pigmentation. Jackson and Burrows, (1956) showed that virulent strains of Y. pestis when grown on defined medium can absorb certain exogenous, planar low-molecular weight chromatophores including hemin and appear as pigmented colonies. Pigmentation is lost on enriched media containing hemin, however, congo red dye is strongly absorbed by virulent

cells on commercial media (Surgalla and Beesley, 1969). Mutants unable to produce pigment show reduced virulence which can be overcome by the injection of mice with iron. The pigmented phenotype appears to be a means of sequestering iron from the environment and storing it internally as inorganic iron (Brubaker, 1984).

Pesticin. Y. pestis produces pesticin (Ben-Gurion and Hertman, 1958), a bacteriocin active against certain colicin-indicator strains of E. coli. Pesticin has a molecular weight of 63,000 daltons and is found in the cytoplasm. It acts by hydrolyzing the cell wall of sensitive bacteria via the enzyme N-acetylglucosaminidase (Ferber and Brubaker, 1979), and converts these bacteria into non-viable osmotically stable spheroplasts (Hall and Brubaker, 1978).

V and W antigens. The V and W antigens discovered by Burrows and Bacon, (1956) are encoded by the 47 Mdal plasmid (Vwa). W is a lipoprotein with a molecular weight of 145,000 daltons while V is a protein of 90,000 daltons (Lawton et al, 1963). The size of V was later reduced to 38,000 daltons suggesting that V exists as either a dimer or in close association with another protein (Straley and Brubaker, 1981). The V and W antigens are restricted to the cytoplasmic fraction of Y. pestis (Straley and Brubaker, 1982). This presents a problem in that the plasmid associated W antigen may confer resistance to intracellular digestion of Y. pestis by phagocytes (Bolin et al, 1982) while antibodies against V can passively protect mice from infection by Y. pestis (Lawton et al, 1963).

Virulence Plasmid

The three species of Yersinia which are pathogenic for humans all

contain plasmids. Y. pestis harbors three characteristic plasmids: the 6 Mdal plasmid which encodes for pesticin production as well as coagulase and fibrinolysin, the 65 Mdal plasmid which is cryptic with respect to humans but which encodes for the murine toxin, and finally a 47 Mdal plasmid which encodes for the production of V and W antigens as well as calcium dependency for growth at 37°C (Ferber and Brubaker, 1981), and a series of outer membrane proteins (Portnoy and Martinez, 1985). It is the 47 Mdal plasmid (Vwa) which is found in all three species of Yersinia. The Vwa plasmid from Y. pestis and Y. entercolitica share approximately 55% DNA homology distributed over 80% of the plasmid genome (Portnoy and Falkow, 1981) while the plasmids associated with Y. pestis and Y. pseudotuberculosis are virtually identical (Portnoy et al, 1984). The region of the plasmid responsible for calcium dependency is highly conserved in all three species, however, the physiological expression is not identical and is more pronounced in Y. pestis (Carter et al, 1980).

Calcium dependency (Lcr). Virulent strains of Y. pestis become attenuated upon serial passage at 37°C in aerated broth (Devignat and Schoetter,1942; Fukui et al, 1957). Kupferberg and Higuchi (1958) demonstrated that growth of virulent cells at 37°C with aeration was dependent on the presence of calcium. Higuchi et al (1959) refined this observation and showed that virulent cells remain static at 37°C in the presence of magnesium. Stasis could be alleviated, however, by the addition of calcium, zinc, or strontium. Avirulent cells do not express this requirement and the calcium requirement is not expressed by either cell type at 26°C. This led to the development by Higuchi and Smith (1961) of a solid medium containing magnesium (20mM) and oxalate (20mM) which is

selective for avirulent mutants at 37°C. These mutants were found to arise at a mutation rate of 10^{-4}/ cell generation.

The bacteriostatic or restrictive condition seen in Y. pestis is characterized by several morphological changes, the most obvious of which is that the cells elongate but do not divide. Cells in bacteriostasis do show septation of the cell membrane, but not of the cell wall. Yang and Brubaker (1971) demonstrated that the rate of DNA synthesis gradually decreases during stasis with total cessation at about 4 h after withdrawal of calcium. During this time the cells complete any current round of chromosome replication. Brubaker (1972) also demonstrated that cells in stasis for 6 h contain at least twice the number of visible nuclei when compared to dividing cells. Cells which are grown under restrictive conditions contain nucleoids that appear as axial filaments, while those grown under permissive conditions have a lobate structure (Hall et al, 1974).

In Y. pestis, transfer of cultures grown at 26°C to a medium deficient in calcium at 37°C results in a cessation of growth (no further increase of cell biomass) after two to four divisions (Zahorchek et al, 1979). This stasis is a result of a downshift with respect to anabolic functions; the percentage of DNA and protein per unit cell mass increasing at the same ratio, while the RNA ratio decreases. Static cells also exhibit a reduction in the adenylate energy charge from 0.8 to 0.6, as well as a reduction in the ribonucleoside triphosphate pools. The deoxyribonucleotide pools remained constant.

Charnetzky and Brubaker (1982) demonstrated that a decrease in the production of stable RNA is associated with the restrictive condition and that unstable RNA (mRNA) has a longer half life than that associated with the unrestricted condition. However, those regulatory nucleotides normally

seen in the stringent response and in the decrease of stable RNA, guanosine tetraphosphate and guanosine pentaphosphate, exhibit a transient rise in levels but no sustained increase. The reduction of RNA synthesis was not a result of stringent response.

YOP. The 47 Mdal plasmid encodes for a series of outer membrane proteins termed Yersinia outer membrane proteins (YOP). Like the Lcr response, these proteins appear when the bacterium is grown at 37^{o}C in the absence of added calcium (Portnoy and Falkow,1981; Martinez, 1983). Several of the plasmid encoded YOP from Y. entercolitica and Y. pseudotuberculosis have identical molecular weights and also cross react antigenically. Y. pestis, it was thought, did not express these polypeptides at either temperature (Portnoy et al, 1984; Straley and Brubaker,1981). Recent evidence suggests that the plasmid-mediated peptides, including the V antigen, are produced in Y. pestis but are posttranslationally degraded by a protein encoded by the 6 Mdal plasmid (Sample and Brubaker, 1987). Evidence for this is seen in that strains, which do not carry the 6 Mdal plasmid, do express these polypeptides at 37^{o}C. This observation would explain why mice infected with Y. pestis generate antibodies to some Y. enterocolitica outer membrane proteins (Portnoy and Martinez, 1985). This is also supported by the fact that when the Vwa plasmid is placed into a cured strain of Y. pseudotuberculosis, YOP were expressed (Wolf-Watz et al, 1985).

The genes associated with the lcr region (including V) and the production of YOP span approximately 70% of the Vwa plasmid (Straley and Bowmer, 1986; Perry et al,1986). In addition, the production of V and YOP are controlled in a coordinate fashion under the influence of the low calcium response genes (Perry et al, 1987).

Plasmid Incompatibility

The phenomenon of plasmid incompatibility was first recognized by Maas and Maas (1962). Plasmid incompatibility is usually defined as the failure of two plasmids to coexist in the same cell line in the absence of external selection (Novick et al, 1976). Incompatibility is due to the sharing of one or more elements of plasmid replication or partitioning by two plasmids (Novick 1987). Plasmid incompatibility can be further defined as symmetric (either plasmid is lost with equal probability) or vectorial (one plasmid is lost exclusively or at a higher rate). Conjugative and non-conjugative plasmids are currently classified into over twenty five separate groups.

It has been generally assumed that plasmids of the same incompatibility group share a phylogenetic relatedness. In general, plasmids belonging to the same incompatibility group have similiar molecular weights (Jacob et al, 1977), for example, plasmids of the Inc W group are from 25-30 Mdal, Inc F1 plasmids are around 60 Mdal, while Inc H plasmids are often over 100 Mdal. Plasmids usually exhibit surface exclusion specifically towards other plasmids of the same incompatibility group. The presence of a particular antibiotic resistance gene shows little correlation to incompatibility group (due to their transposon mediated nature) (Datta, 1979).

There is however, a broad correlation between pilus type and certain incompatibilty groups. Plasmids of the Inc F group were called so because they all determine F pili (Meynell et al, 1968) that show serological and physical similarities. The same phenomenon exists in virtually each Inc

group; that of serologically distinct pilus formation between groups and cross reaction within a given group. There exist three basic morphological forms of pili: thin and flexible; thick and flexible; and rigid (Bradley, 1980).

Closely related plasmids within a given incompatibility group show significant seqence homology. This is generally true of the conjugative plasmids and their homology is attributed to significant similarity in those genes which govern pilus synthesis and transfer functions. There are exceptions to this rule, notably those plasmids of the Inc H1 and Inc H2 groups which do not show sequence homology to any significant extent (Grindley et al, 1973).

II. MATERIALS AND METHODS

Bacterial Strains and Growth Conditions

Bacterial strains used in this study are listed in Table 1. All Yersinia strains are attenuated by virtue of being non-pigmented. E.coli was grown at 37°C with aeration, diluted, and plated on Luria-Bertani (LB) media (Maniatis et al, 1982A). Y. pestis was grown at 26°C with aeration in Heart Infusion Broth (Difco Laboratories, Detroit, Mich). The selective agents used were ampicillin (Ap), chloramphenicol (Cm), kanamycin (Kn), nalidixic acid (Nal), sodium azide (Azi), streptomycin (Sm), and tetracycline (Tc) (Sigma Chemical Co., St. Louis, MO.) and were used at final concentrations of 50,25, 50, 40, 250,100 , and 12.5 ug/ml respectively. Sugar utilization was determined by overnight growth on MacConkey basal agar (Difco Laboratories, Detroit, Mich) supplemented with the appropriate sugar to a final concentration of 1% or by overnight growth on Violet Red Bile Agar (VRB).

Transduction,Transformation

Those strains with origins designated "this study" in Table I were derived by transduction of the Vwa plasmid (pYV019::Tn5) of Y. pestis from E. coli LE392 by phage P1 $_{vir}$ (Miller, 1977A). Lysates were prepared by diluting an overnight culture into fresh LB broth supplemented with $5x10^{3}$M

TABLE 1. BACTERIAL STRAINS

Strain	Plasmid Content	Relevant Genotype	Reference/ Source
Escherichia coli			
HfrC	F	met.T_2^r	C. Thorne
HfrC	HfrC/pYV019::Tn5		this study
HB101	-	hsdS20(r^-_B,m^-_B), recA13.ara-14.proA2, lacY1.galK2.rpsL20, xyl-5.mtl-1.SupE44	H. Boyer
J53	-	pro.met.thi	E. Meynell
J53-1	-	pro.met.thi.nal^r	E. Meynell
J53-2	-	pro.met.thi.rif	E. Meynell
J E2571	-	leu.thr.Sm^r.fla.pil	D. Bradley
K57	F'Cm		W. Lawton
K57	F'Cm/pYV019::Tn5		this study
LE392	-	hsdR514(r^-_K,m^+_K) SupE44.Sup F58.galK2, Δ(lacIZY).galT22, metB1.trpR55	S. Falkow
LE392	pYV019::Tn5		S. Falkow
SE5000	-	araD139.Δ(argF-lac) U169.rpsl150.relA1, recA56.deoC1.ptsF25, rbsR.flbB5301	G. Weinstock
SE5000	F'Cm/pYV019::Tn5		this study
WIA2	-	lac.Nal^r,Azi^r	P. Guyre
WIA2	F'Cm/pYV019::Tn5		this study
W1177	-	thr.leu.thi.lac.ara. gal.mal.mtl.xyl.$T1^r$, T_6^r,Sm^r	C. Thorne
23-10S	F'lac	met	W. Lawton
Yersinia pestis			
019	Vwa	lac.pgm	S. Straley
EV7651F	61,47,6	lac.pgm	D.Cavanauh
EV7651F-5.5	61,47,6,RSF1030	lac.pgm	R.M.Zsigray

calcium chloride and grown to an approximate density of $2x10^8$ cells/ml. Approximately 10^7 $P1_{vir}$ were added to 1 ml of this culture and the mixture incubated at 37°C for 20 min. The culture was added to R-top soft agar and poured on a R-top plate, (R-top agar is per liter: 10 g tryptone, 8 g sodium chloride, 1 g yeast extract, 15 g agar) and incubated at 37°C for 8 h. The soft agar was scraped from the plate, placed in a centrifuge tube, several drops of chloroform added, and the sample vortexed vigorously. The agar was pelleted by centrifugation and the supernate containing the $P1_{vir}$ lysate was recovered.

Transduction involved the resuspension of an overnight culture in MC buffer (0.1 M magnesium sulfate, 0.005 M calcium chloride), incubation at 37°C with aeration for 15 min , and addition of P1 lysate. P1 was preadsorbed by incubation at 37°C for 20 min and further infection was prevented by addition of 1M sodium citrate. Cells were then plated on medium containing the appropriate inhibitors and incubated for 24 h at 37°C.

Transformation of E. coli was by the calcium chloride procedure (Mandel and Higa 1970, Maniatis et al,1982B). An overnight culture was diluted 1:100 into 40 ml of LB broth and the culture incubated at 37°C for 3h with vigorous aeration. The culture was centrifuged at 4000xg for 5 min at 4°C. Cells were resuspended in an ice cold, sterile solution of 50mM calcium chloride in half the original culture volume and held at 0°C for 30 min. At this point the cells were again centrifuged at 4000xg for 5 min and resuspended in 50mM calcium chloride (1:20 of the original culture volume). After a second 30 min incubation at 0°C the cells were considered competent. Upon addition of DNA, the mixture was incubated for 10 min at 0°C followed by a 2 min incubation at 42°C. Subsequent to the addition of

1 ml of prewarmed LB broth, the suspension was incubated at 37°C for 20 min and plated on medium containing the appropriate selective inhibitors and incubated for 17-24 h at 37°C.

Conjugation and Phage MS-2 Testing

Unless otherwise stated, matings were for 30 min using the procedure described by Miller (Miller, 1977B). Overnight cultures of the donors were diluted 1:40 into fresh LB broth and incubated at 37°C for 3 h with gentle agitation. At this point, equal volumes of donor and overnight recipient cultures were mixed at 37°C. Mating pairs were disrupted by vigorous vortexing for 30 sec, diluted and plated.

Phage testing was by the spot method of Miller (Miller ,1977C). Cells to be tested were mixed with soft agar, and poured on an LB agar plate. Upon solidification, phage (MS-2) was spotted on the plate and allowed to dry. Incubation was for 24 h at 37°C.

Plasmid Isolation for Agarose Gel Electrophoresis

Plasmids used in this study are listed in Table 2. Plasmid DNA was isolated for agarose gel electrophoresis using the alkaline lysis method (Birnboim and Doly, 1979). After centrifugation (10,000xg for 5 min) of 3 ml of an overnight culture, the cells were resuspended in 90 ul of wash buffer (50mM glucose, 25mM Tris-HCl, 10 mM EDTA, [pH8.0]). Bacterial cell walls were weakened by the addition of lysozyme to a final concentration of 4 mg/ml. Following 30 min at 0°C, cells were lysed by the addition of 200 ul lysis solution (0.2N sodium hydroxide, 1% sodium lauryl sulfate [pH 12.5]). After 5 min at 0°C, the chromosomal DNA was irreversibly precipitated by

TABLE 2. PLASMIDS

Plasmid	Bacterial Host	Incompatability Group	Molecular Weight(Mdal)	Reference/ Source
R16	J53	B	69	D. Bradley
RA1	J53-1	C	86	T. Aoki
F'Cm	K57	F	-	W. Lawton
R64	JE2571-1	I_1	72	D. Bradley
R621a	JE2571	Igamma	65	R. Hedges
R27	JE2571	HI1	112	D. Bradley
pHH1508a	JE2571	HII	100	D. Bradley
R387	J53	K	53	D. Bradley
R446b	J53-1	M	47	R. Hedges
N3	J53	N	33	D. Bradley
RP4	JE2571	P	36	D. Bradley
R402	J53-1	T	86	J. Coetzee
RA3	J53	U	30	D. Bradley
Tp231	J52-2	X	36	D. Bradley
Sa	J53	W	25	N. Grindley
pGC1	JC3272	-	33	G. Cornelis
pBR328	HB101	-	3.23	X. Soberon

the addition of 150 ul of potassium acetate (5M potassium acetate [pH 4.8]). Precipitated chromosomal DNA was removed after a 60 min incubation at 0°C by centrifugation and the supernate containing the plasmid was mixed with two volumes of ethanol. Plasmid DNA was precipitated by centrifugation after the preparation was held at -70°C for 20 min or -20°C for 60 min and the pellet resuspended in 50 ul TE buffer (10mM Tris-HCl, 1mM EDTA [pH 8.0]) (Crosa and Falkow, 1981). Plasmid DNA was applied to either a vertical (12cmx14cmx3mm) or a horizontal (10cmx15cm or 5cmx15cm) 0.8% agarose gel. Electrophoresis was for 3 h at 90 V for the vertical gels and 60 V for 2.5 h for the horizontal gels in a Tris-acetate buffer (0.04M tris, 0.02M sodium acetate, 2mM EDTA [pH 7.8]). Prior to electrophoresis, plasmid samples were mixed with a tracking solution (33% glycerol, 0.07% bromphenol blue, 7% sodium lauryl sulfate) in a sample to solution ratio of 3:1. Gels were stained with ethidium bromide (1ug/ml) and viewed with a model C-63 transilluminator (Ultra-violet Products, Inc.)

Plasmid Isolation for Restriction Endonuclease Analysis

Plasmid DNA was isolated by the alkaline lysis method. After ethanol precipitation and centrifugation the pelleted DNA was resuspended in TE buffer and treated with RNAse at a final concentration of 1mg/ml for 15 min at 4°C. Deproteinization was achieved by the addition of an equal volume of Tris equilibrated phenol, followed by vortexing, centrifugation, and removal of the bottom phase. An equal volume of chloroform-isoamyl alcohol (24:1),was then added, followed by vortexing, centrifugation, and removal of the bottom phase. Plasmid DNA was precipitated by the addition of 2 volumes ethanol at -70°C for 20 min (Maniatis et al, 1982C) followed by

centrifugation. After resuspension in the appropriate core buffer, plasmids were digested with restriction endonucleases (Bethesda Research Laboratories, Bethesda MD) according to manufacturer's instructions.

Photography

Gels were photographed using Polaroid Type 55 positive/negative film in combination with a Kodak gelatin filter #16. Exposures were for 3 min, f-stop set at 4.7, shutter speed at B, with the lens at various distances from the gel.

Plasmid Isolation and ^{32}P Labeling of Probe DNA

Plasmid DNA was isolated from Y.pestis 019, which contains only the Vwa plasmid, as previously described (Zsigray et al, 1985). Briefly, the procedure described by Birnboim and Doly (Birnboim and Doly, 1979) was used with reagent volumes increased proportionately as required for isolation of the DNA from 1 liter of cells grown overnight at 26°C with aeration in Heart Infusion Broth. After ethanol precipitation, and centrifugation, the pelleted DNA was dried under vacuum and the pellet redissolved in 7 ml TE buffer.

The solution of plasmid DNA was added to a tube containing 9g of CsCl in 2 ml distilled water. Upon dissolution of the CsCl, 0.96 ml of ethidium bromide (10mg/ml) was added and the mixture was centrifuged in a Beckman 50 Ti rotor at 183,000xg for 40 h at 20°C. The plasmid band was recovered and the ethidium bromide extracted several times with equal volumes of isoamyl alcohol. The plasmid DNA was then dialyzed against standard saline-citrate (pH 8.0).

Plasmid DNA was labelled in vitro with [α-32P] d CTP (New England Nuclear Corp., Boston, Mass.) by nick translation (Maniatis et al, 1975) Labeled DNA was separated from unincorporated radionucleotides by passage of the mixture across a Sephadex G-50 matrix equillibrated with STE buffer (10 mM Tris-HCl, 100mM sodium chloride, 1mM EDTA[pH 8.0]) (Maniatis, 1982D). The sample was centrifuged at 1,600xg for 4 min. The labeled probe was found in the eluate.

Southern Blotting

Southern filter hybridization (Southern, 1975) was used to detect homologous sequences common to the Vwa plasmid and those conjugative plasmids of various incompatibility groups listed in Table 2. Unless otherwise stated, all gel manipulations were at room temperature with gentle agitation. To insure transfer of high-molecular-weight DNA to Trans-Blot membranes (Bio-Rad Laboratories, Richmond, CA), gels were first treated with 0.25 M HCl. Southern transfers were achieved using the methods described by Maniatis et al (1982D). Prior to alkaline denaturation, gels were neutralized by soaking in several volumes of 1M tris buffer (pH 8.0) for 30 min. Alkaline denaturation was accomplished by soaking the gel for 60 min in 0.5M sodium hydroxide and 1.5M sodium chloride, followed by a second exposure to Tris buffer. Transfer buffer consisted of 10X SSC (per liter: 87.7g sodium chloride, 38.7g sodium citrate[pH 7.0]) and Whatman 3MM filter paper was used as the wick. DNA transfer to the nitrocellulose membrane was due to the capillary action brought about by the combination of a stack of paper towels topped by a 500g weight. Transfer was allowed to proceed for 17-24 h. Following transfer, the membrane was soaked in 6X SSC for 5 min, dried, and baked in a vacuum oven at 80^{o}C for 2 h. The

nitrocellulose was stored at room temperature under vacuum.

Hybridization

The nitrocellulose filter was soaked in 6X SSC for 2 min, placed in a heat sealable bag, and exposed to 0.2 ml of prehybridization fluid per cm^2 (6X SSC, 0.5% SDS, 5X Denhardt's solution, and 100 ug/ml denatured salmon sperm DNA [Denhardt's solution was prepared as a 50X stock of 5g Ficoll, 5g polyvinylpyrrolidone, 5g bovine serum albumin, in 500 ml water.]). The filter was incubated at 68°C for 2-4 h. At this time the prehybridization fluid was removed and replaced with hybridization fluid (hybridization fluid was the same as prehybridization fluid plus the addition of 0.01M EDTA as well as the radioactively labeled probe DNA) at 50 ul/cm^2 and hybridization was for 18 h at 68°C. The filter was then washed at room temperature in 2X SSC, 0.5% SDS for 5 min. This was followed by a second wash that consisted of 2XSSC, 0.1% SDS. Finally the filter was washed in 0.1X SSC, 0.5% SDS for 2h at 68°C. The filter was then dried at room temperature.

Autoradiography

Sequence homology was visualized by the exposure of the nitrocellulose filter to Kodak X-Omat Ar-5 X-ray film. The filter was wrapped in plastic wrap, and taped to the X-ray film. The film was placed between two intensifying screens (Dupont Cronex Lightning-plus) in a Kodak X-ray exposure holder. Incubation was for various times at -70°C. The X-ray film was developed using Kodak GBX developer for 3 min followed by 10 sec in a stop bath (10% acetic acid) and 10 min in Kodak fixer.

Construction of Recombinant Plasmids

Vwa plasmid DNA was isolated from Y. pestis 019 and pBR328 from E. coli HB101(Soberon et al, 1980). Plasmids were digested with the restriction endonuclease BamH1 (Bethesda Research Laboratories, Bethesda, MD). The two plasmids were mixed and an approximate total of 5 ug of plasmid DNA was combined in a 2:1 insert to vector ratio with respect to pmol ends. The fragments were covalently joined using T4 DNA ligase (Bethesda Research Laboratories, Bethesda, MD) and the plasmids used to transform E. coli HB101 (F'lac). Cells were plated on LB agar containing chloramphenicol and ampicillin.

III. RESULTS

Mobilization of the Vwa Plasmid

This study originally began as an extension of the work reported by Zsigray et al (1983,1985), who showed that the transfer of the fertility factor (F) of Escherichia coli to Yersinia pestis resulted in a loss of virulence. This avirulence was attributed to the loss of calcium dependence and the plasmid profiles of Y. pestis strains harboring F, in combination with Southern blots, revealed that the 47 Mdal plasmid of Y. pestis had integrated into the Y. pestis chromosome in those cells harboring F. A plausable explanation for this phenomenon was plasmid incompatibility.

The question of whether a similiar phenomenon existed in E. coli strains harboring both F and the Vwa plasmids was investigated. E. coli K57 (F'Cm) was mated with E. coli LE392 (pYV019::Tn5) and the transconjugants were used in additional matings to determine the degree of sexduction (F'Cm transfer) or chromosomal mobilization displayed by each isolate. Every transconjugant transferred the F'Cm at a high frequency while chromosome mobilization was a rare event, suggesting that F remained as an autonomous, cytoplasmic element in cells containing pYV019::Tn5.

The 6 Mdal plasmid has been reported to be mobilized by strains of Y. pestis that harbor an R factor (Kol'tsova et al, 1971). These results prompted me to determine if the two remaining plasmids associated with Y. pestis might likewise be mobilized. Consequently, the pYV019::Tn5 was transduced to several F-containing strains of E. coli (Table 1) by $P1_{vir}$.

Escherichia coli K57 (F'Cm/pYV019::Tn5) was mated with E. coli WIA2

and the mixture plated on a medium that selected for transconjugants receiving both plasmid markers. Agarose gel electrophoresis of plasmids isolated from the transconjugants verified that both the fertility factor of E.coli and pYV019::Tn5 were cotransferred to recipient cells (Fig. 1). Additional matings using E. coli K57 (F'Cm/pYV019::Tn5) and WIA2 were performed using various selective conditions to elucidate the mechanism(s) of transfer of the Vwa plasmid (Tables 3,4). Twenty-seven transconjugants (9 from each selection), were tested for the recipient phenotype and all proved to be lactose negative and sodium azide resistant, indicating that the Vwa plasmid was mobilized (transferred) in the F-containing strains. All transconjugants selected for F, as well as those selected for the presence of both plasmids, were MS-2 sensitive. The 9 transconjugants selected on kanamycin were also chloramphenicol resistant; however, none of those selected on chloramphenicol exhibited kanamycin resistance. The plasmid profiles of 4 transconjugants selected for kanamycin resistance and 2 for chloramphenicol resistance are shown in Fig. 2A. In most cases the Vwa plasmid was transferred along with F. Overall, the frequency of Vwa mobilization was approximately 10^{-3} when expressed as a percent of F transfer. The presence of pYV019::Tn5 in the transconjugants was confirmed when the plasmid DNA was transferred to nitrocellulose filters and the DNA probed with the 47 Mdal plasmid of Y. pestis 019 (Fig. 2B).

Final confirmation of the mobilization of the Vwa plasmid was obtained when several transconjugants from the previous experiment were employed as donors in a cross with E. coli W1177 (Table 5). The Vwa plasmid was seen in the plasmid profiles and again was confirmed by Southern hybridization (Fig. 3A, 3B). In this mating the frequency of Vwa

Fig. 1. Plasmid profiles of transconjugants obtained from a mating of E. coli K57(F'Cm/pYV019::Tn5) and E. coli WIA2. Selection was LB agar supplemented with chloramphenicol, kanamycin, and nalidixic acid. Lanes 1,4-5,7-9=transconjugants; lane 2=Y.pestis EV7651F; lane 3=E.coli K57; lane 6=Y.pestis EV7651F containing the E.coli plasmid RSF 1030; lane 10=E.coli WIA2.

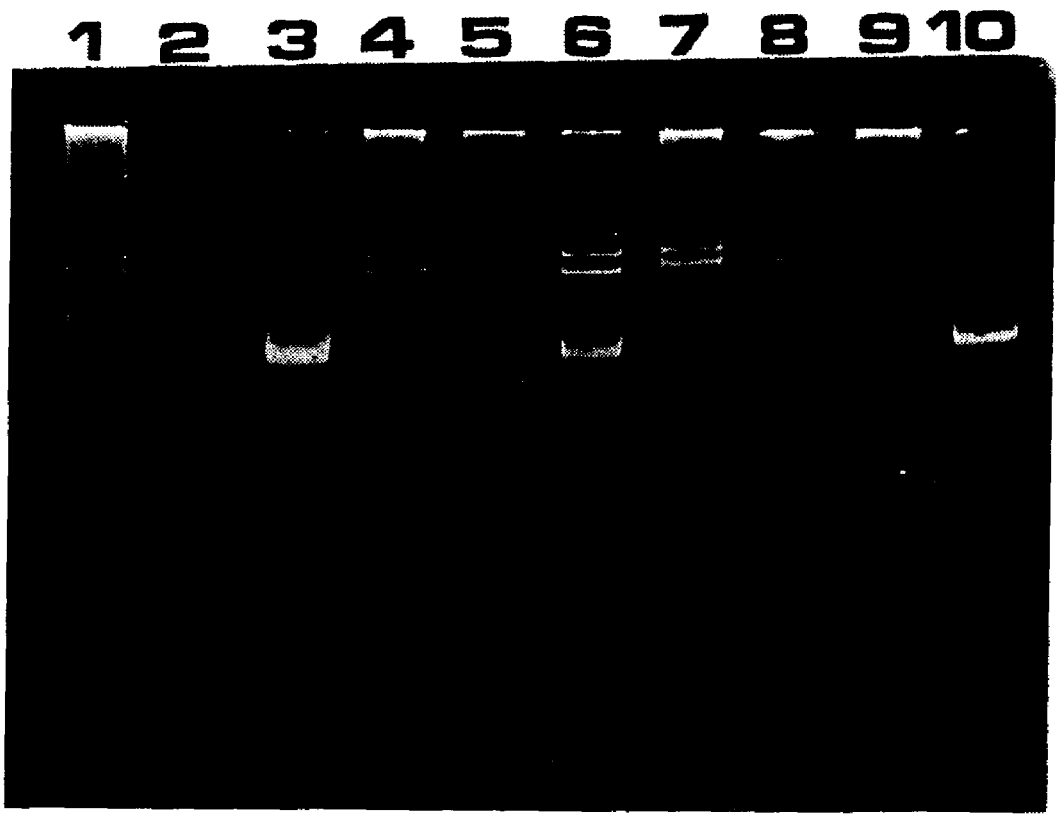

1 2 3 4 5 6 7 8 9 10

Table 3.

Mating of E. coli K57(F'Cm/pYV019::Tn5) X E. coli WIA2

Selection	% Transconjugants[a]	% Transconjugants[b]
Cm,Nal	20.2	100
Kn,Nal	1.8×10^{-4}	8.7×10^{-4}
Kn,Cm,Nal	2.6×10^{-4}	1.3×10^{-3}

a=expressed as percent of donor input
b=expressed as percent of F transfer

Table 4

Characterization of Transconjugants from mating in Table 3

Transconjugants	Lactose	Azi^r	MS-2	Antibiotic^r
Kn,Nal-9 isolates	all lac$^-$	all azi^r	NT	all Cm^r
Cm,Nal-9isolates	all lac$^-$	all azi^r	all MS-2^s	none Kn^r
Kn,Cm,Nal-9isolates	all lac$^-$	all azi^r	all MS-2^s	all Kn^r,Cm^r

NT=not tested

Fig. 2. A. Plasmid profiles of transconjugants from additional matings of E.coli K57 (F'Cm/pYV019::Tn5) and E.coli WIA2. Selection was for chloramphenicol and nalidixic acid (lanes 2 and 3); kanamycin, chloramphenicol, and nalidixic acid (lanes 5 and 6); kanamycin and nalidixic acid (lanes 8 and 9). Lane 1=E.coli K57; lane 4=Y.pestis EV7651F containing the E.coli plasmid RSF 1030; lane 7= E.coli LE392; lane 10=E.coli WIA2. B. Southern hybridization of gel in (A) using the Vwa plasmid of Y.pestis 019 as probe

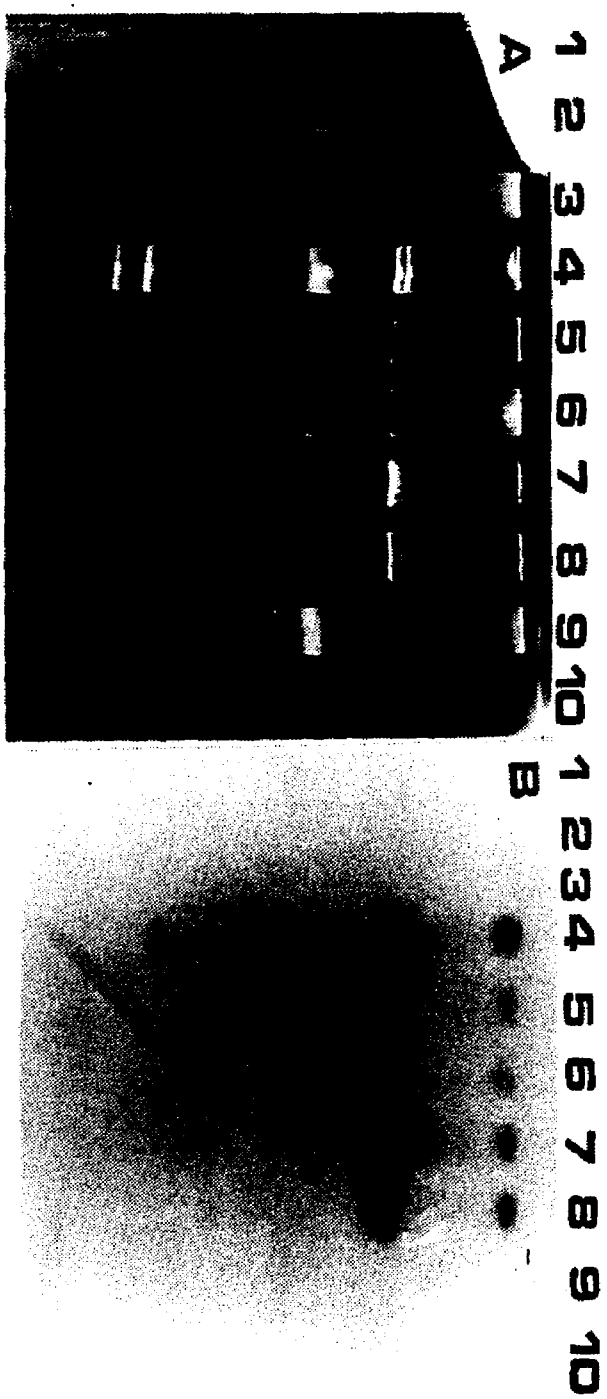

A
1 2 3 4 5 6 7 8 9 10
B
1 2 3 4 5 6 7 8 9 10

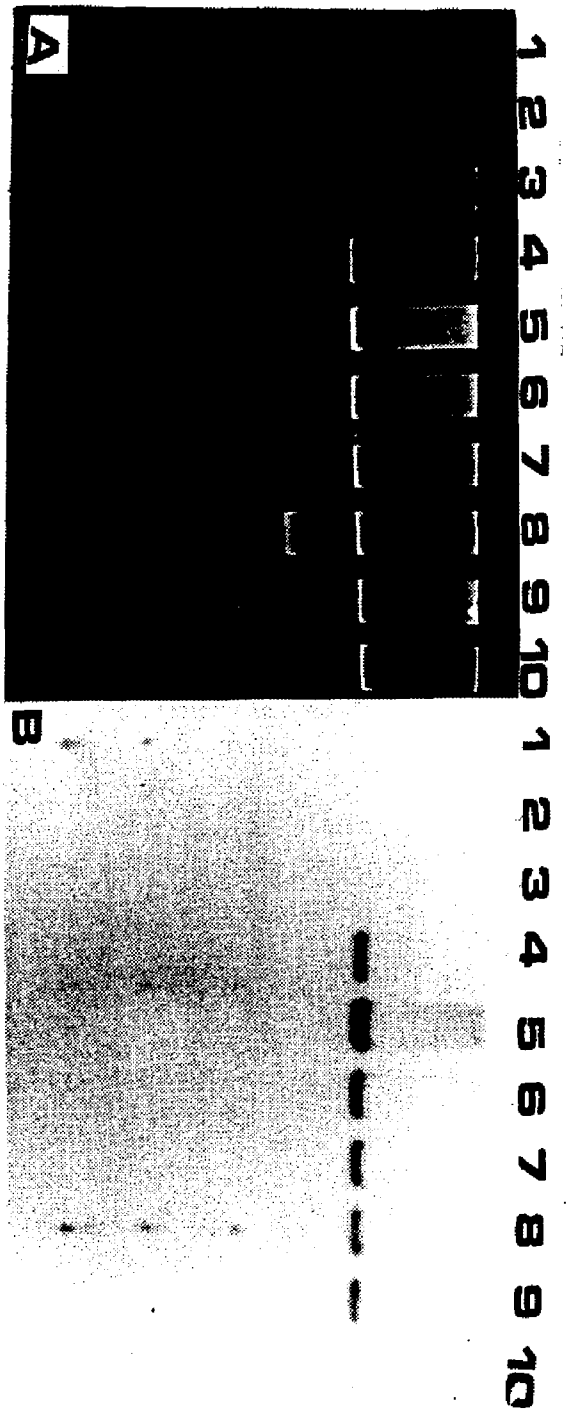
Table 5

Mating of E. coli WIA2 (F'Cm/pYV019::Tn5) X E.coli W1177

Selection	% Transconjugants[a]	% Transconjugants[b]
Cm,Sm	.21[c]	100
Kn,Sm	2.3×10^{-4}	1.1×10^{-1}
Kn,Cm,Sm	1.2×10^{-4}	5.6×10^{-2}

a=expressed as percent of donor input
b=expressed as percent of F transfer
c=mating terminated after 10 minutes

Fig 3. A. Plasmid profiles of transconjugants from a mating of E.coli WIA2 (F'Cm/pYV019::Tn5) and E.coli W1177. Selection was on media containing chloramphenicol and streptomycin (lanes 1-3), chloramphenicol, kanamycin, and streptomycin (lanes 5-9). Lanes 4 and 10=E.coli LE392. B. Southern hybridization of gel in (A) using the Vwa plasmid of Y.pestis 019 as probe.


A
1 2 3 4 5 6 7 8 9 10
B
1 2 3 4 5 6 7 8 9 10

mobilization was on the order of 10^{-1} to 10^{-2} with respect to F. It should be noted that in the transconjugants selected for both plasmids (lanes 5-9) only the pYV019::Tn5 was evident. However, all were MS-2 sensitive, suggesting that an Hfr was established upon cotransfer of F to W1177.

The results obtained at this point could have been interpreted to mean that the mobilization of the Vwa plasmid resulted from cointegrate formation between the two plasmids prior to transfer, especially since the incD region of F shares sequence homology with a portion of the Vwa plasmid (Bakour et al, 1983). The possibility that mobilization of pYV019::Tn5 resulted from recombinant cointegrate formation was eliminated by mating a recA donor containing both plasmids, E coli SE5000 (F'Cm/pYV019::Tn5), with E. coli WIA2 (Tables 6,7). The transconjugants exhibited the recipient phenotypes and the MS-2 sensitivities, the antibiotic resistance patterns, as well as the frequency of mobilization which were consistent with those of the first mating. Again the plasmid profiles in combination with Southern hybridization confirmed the mobilization of the Vwa plasmid (Fig. 4A,B).

Finally, to determine whether or not an autonomous F was required for mobilization of pYV019::Tn5, a mating was performed between E. coli HfrC(pYV019::Tn5) and WIA2. The transconjugants were scored for both chromosome transfer (lac marker) as well as kanamycin resistance. The results are given in Tables 8 and 9. The presence of the Vwa plasmid was confirmed as above (Fig 5A,B). Unexpectedly, the majority of the kanamycin resistant isolates contained a plasmid similiar in size to F. The nature of these particular plasmids awaits clarification.

Table 6

Mating of E. coli SE5000(F'Cm/pYV019::Tn5) X E.coli WIA2

Selection	% Transconjugants[a]	% Transconjugants[b]
Cm,Nal	13.3	100
Kn,Nal	1.2×10^{-3}	9.1×10^{-3}
Kn,Cm,Nal	1.3×10^{-2}	9.8×10^{-2}

a=expressed as percent of donor transfer
b=expressed as percent of F transfer

Table 7

Characterization of Transconjugants from mating in Table 6

Transconjugants	Arabinose	azi^r	Ms-2	Antibiotic^r
Kn,Nal-9isolates	all *ara*$^+$	all *azi*^{r}	all MS-2^S	all Cm^r
Cm,Nal-9isolates	all *ara*$^+$	all *azi*^{r}	all MS-2^S	none Kn^r
Kn,Cm,Nal-9isolates	all *ara*$^+$	all *azi*^{r}	all MS-2^S	all Kn^r,Cm^r

Fig. 4. A. Plasmid profiles of transconjugants from a mating of E.coli SE5000 (F'Cm/pYV019::Tn5) and E.coli WIA2. Selection was on media containing kanamycin and nalidixic acid (lanes 2 and 3), chloramphenicol, kanamycin, and nalidixic acid (lanes 5 and 6), chloramphenicol and nalidixic acid (lanes 8 and 9). Lane 1=E.coli WIA2; Lane 4=E.coli LE392; lane 7=Y.pestis EV7651F containing the E.coli plasmid RSF 1030; Lane 10=E.coli SE5000 (F'Cm/pYV019::Tn5). B. Southern hybridization of gel in (A) using the Vwa plasmid of Y.pestis 019 as probe.

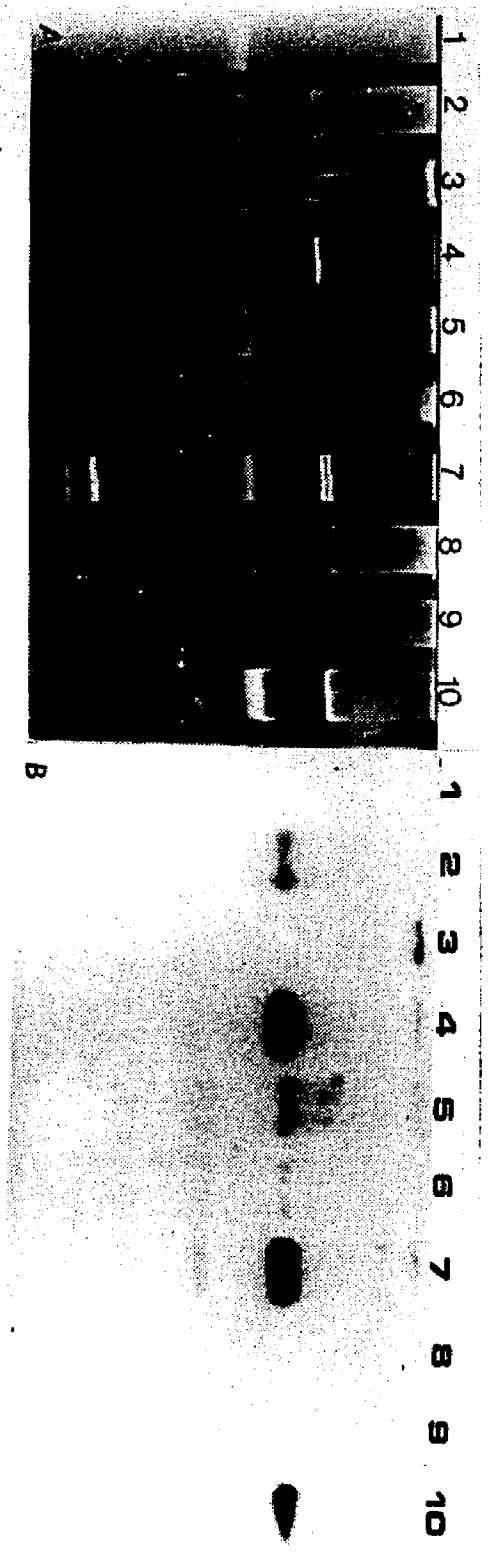

A
1
2
3
4
5
6
7
8
9
10
B
1
2
3
4
5
6
7
8
9
10

Table 8

Mating of E.coli HfrC(pYV019::Tn5 X E.coli WIA2

Selection	% Transconjugants[a]	% Transconjugants[b]
Lactose	1.29	100
Kn,Nal	4.08×10^{-5}	3.17×10^{-3}

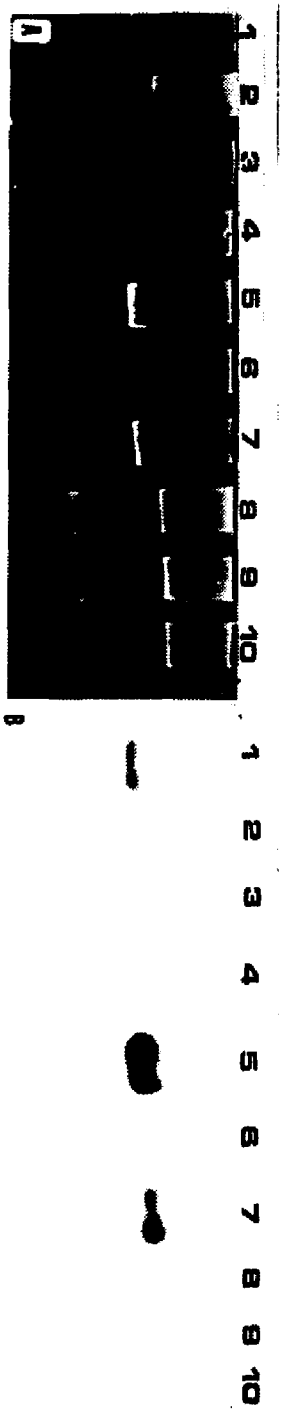
a=expressed as percent of donor input
b=expressed as percent of F transfer

Table 9

Characterization of Transconjugants from mating in Table 8

Transconjugants	azi	lactose
Kn,Nal-9isolates	all azi^r	all lac$^+$

Fig. 5. A. Plasmid profiles of transconjugants from a mating of E.coli HfrC(pYV019::Tn5) and E.coli WIA2. Selection was on media containing kanamycin and nalidixic acid (lanes 1-4 and 7-10). Lane 5=E.coli HfrC(pYV019::Tn5); lane 6=E.coli WIA2. B. Southern hybridization of gel in (A) using the Vwa plasmid of Y.pestis 019 as probe


A
1 2 3 4 5 6 7 8 9 10
B
1 2 3 4 5 6 7 8 9 10

My observations so far had concluded that the Vwa plasmid of Y. pestis was mobilized in the presence of a second conjugative plasmid (F) and that this mobilization was independent of homologous recombination. In addition, mobilization was independent of whether the second plasmid existed as an autosomal cytoplasmic element. Finally, mobilization was not the result of any apparent transposon-mediated cointegrate formation (see discussion).

Cloning of the origin of transfer

My previous observations led me to suspect the Vwa plasmid was mobilized via a possible origin of transfer (ori T). Preliminary experiments to clone the origin of transfer made use of the cloning vector pUC19 (a pBR 322 derivative) and the results indicated the presence of a 5 Kb insert (data not shown). However, since pUC19 contained the pBR 322 nic site, the possibility existed that mobilization occurred via this site. Therefore, the experiments were repeated using the cloning vector pBR 328. To clone and isolate this ori T, the Vwa plasmid from Y. pestis 019 was treated with BamH1, as was the cloning vector pBR328. The restriction endonuclease BamH1 cuts the Vwa plasmid at least 15 times (Fig 6) (Portnoy and Falkow, 1981) as well as cutting pBR328 once in the tetracycline resistance gene (Fig 7) (Soberon et al, 1980). The two plasmids were mixed, ligated, and used to transform E. coli HB101(F'lac), (constructed by mating E. coli 23-10S with E. coli HB101). The ligation mixture was plated onto LB agar containing chloramphenicol, ampicillin, and streptomycin and, after 24 h incubation, over 2,000 clones were isolated. The clones were pooled and used as the donor in a mating with E. coli WIA2. The putative

Fig 6. Restriction map of pYV019 (Vwa) (Portnoy et al, 1982). The lcr region is denoted by the shaded area. The sizes of the BamH1 fragments in Kb's are; 14.3, 10.7, 9.7, 5.8, 5.3, 4.9, 4.4, 4.0, 2.9, 2.2, 1.7, 1.6, 1.3, 1.1, 0.7.

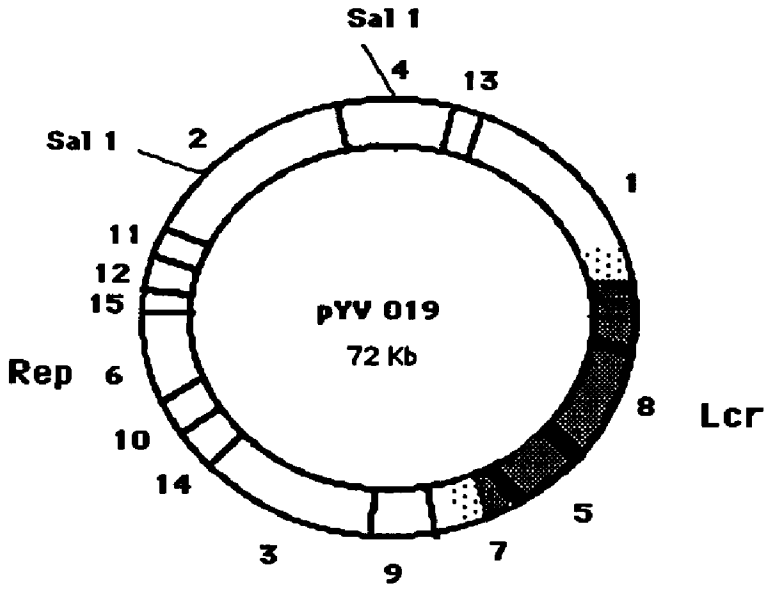
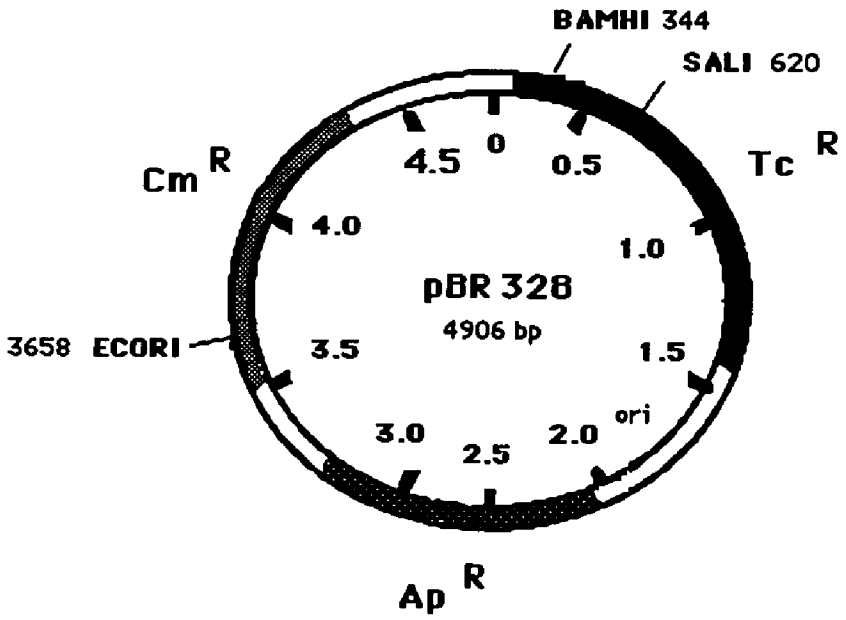

Sal 1
4
13
Sal 1
2
1
11
12
15
pYV 019
72 Kb
Rep
6
8
Lcr
10
14
5
3
7
9

Fig. 7. Restriction map of cloning vector pBR 328 (Soberon et al, 1980). The unique BamH1 cloning site is found in the tetracycline gene.


BAMHI 344
SALI 620
Cm R
Tc R
4.5
0
0.5
4.0
1.0
pBR 328
4906 bp
3658 ECORI
3.5
1.5
ori
3.0
2.5
2.0
Ap R

transconjugants were plated on media that selected for the mobilization of pBR328 (LB agar with Cm, Ap, Nal). The cloning vector pBR328 is a pBR322 derivative in which the nic site has been deleted and as such it cannot be mobilized. The only possible route of conjugal transfer was physical integration into the conjugative plasmid either through homologous recombination to form a cointegrate or through transposon-mediated cointegrate formation. The use of a recombination deficient donor (E.coli HB101) eliminated the first possibility.

The plasmid profiles of the resulting tetracycline sensitive recipients (E.coli WIA2) were determined by agarose gel electrophoresis (Fig 8). The results indicated that 78% (21 of 27) of the mobilized vectors carried an insert of approximately 9.1 Kb, 11% an insert of 8.3 Kb, and11% carried an insert of 7.2 Kb .

To better define the nature of the inserts present in pBr328, the plasmids from several clones (isolates 1,2,3,4,5,7,9,14) were digested with BamH1 and subjected to agarose gel electrophoresis (Fig 9). In each case the digestions indicated the presence of pBR328. Isolate #1 showed a fragment similiar to the BamH1-5 fragment of Vwa plasmid. Isolates 2,3,5,7, and 9 indicated the presence of two fragments, the BamH1-4 and BamH1-9 fragments of the Vwa plasmid. Isolate 4 gave an unusual pattern of BamH1-10 and a second fragment larger than the BamH1-4 fragment but smaller than the BamH1-3 fragment. Isolate 14 also indicated the presence of this aberrant fragment. It was possible that the unusual restriction pattern obtained with isolates 4 and 14 was the result of the presence of gamma-delta sequences (Tn 1000) derived from the F plasmid, since an earlier report which dealt with the mobilization of pBR322 by an F plasmid

Fig 8. Plasmid profiles of E. coli WIA2 carrying the mobilized plasmid pBR328. Lanes 10 and 20=plasmid pBR328; Lane 15=plasmids from Y. pestis EV7651F; Lanes 1-9=isolates 1-9; Lanes 11-14=isolates 10-13; Lanes 16-20=isolates 14-17; Lanes 21-30=isolates 18-27.

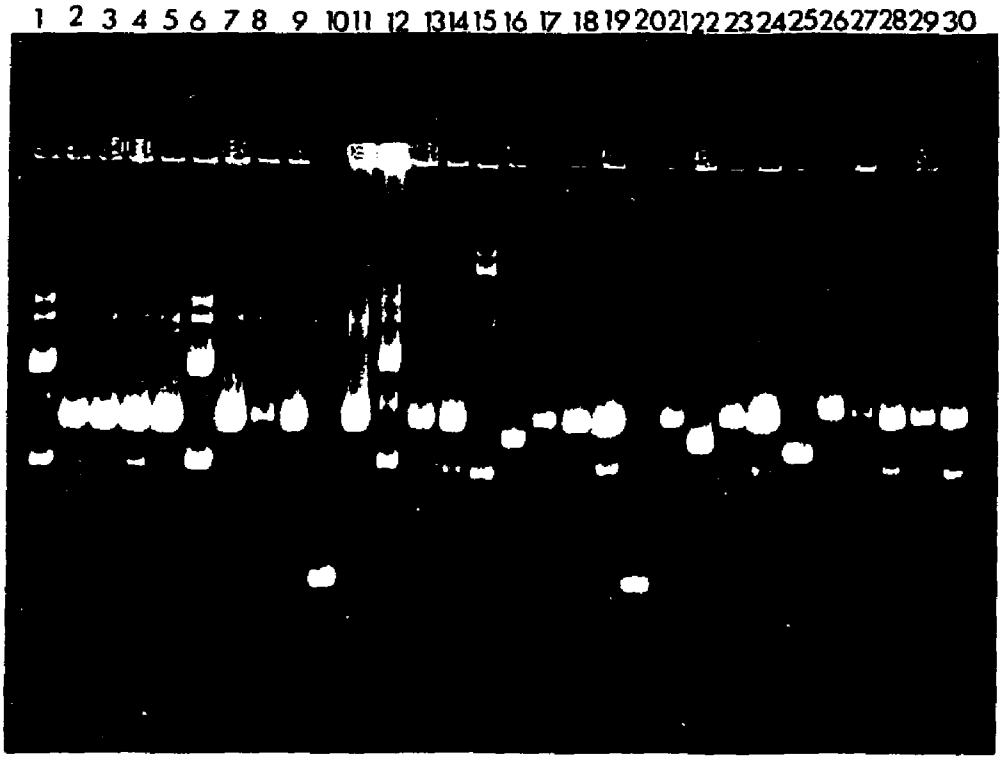
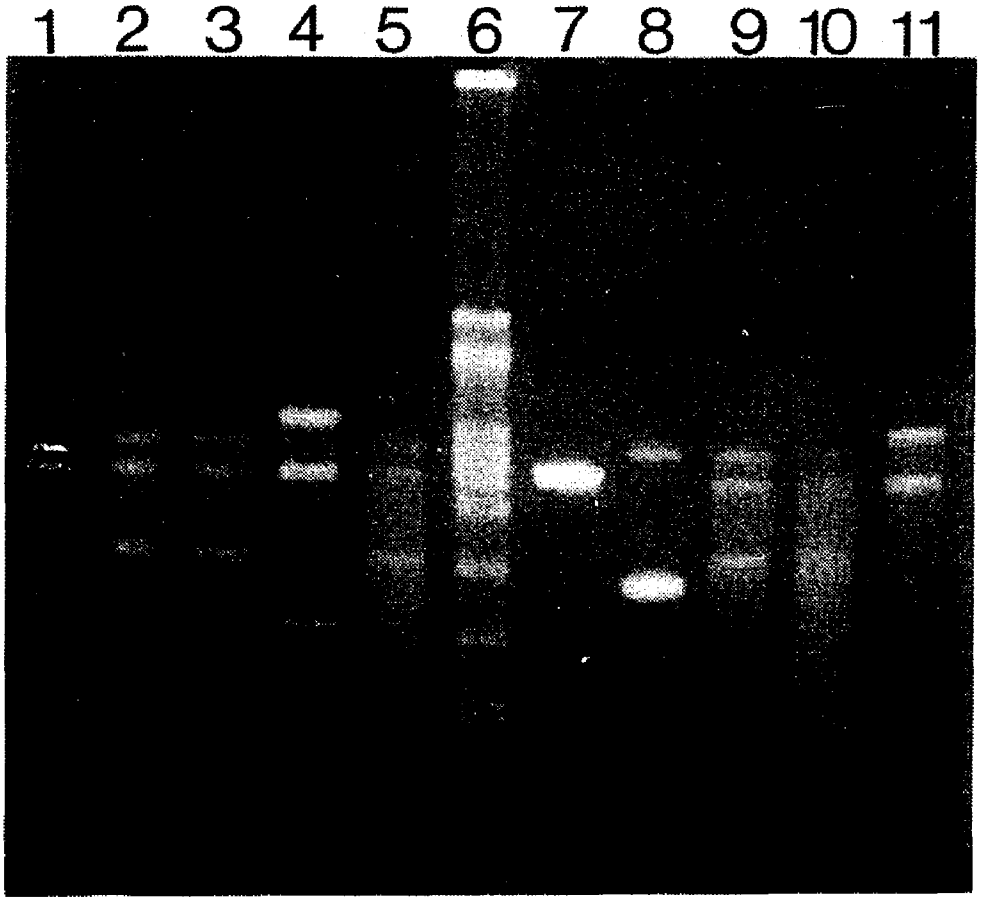

1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27 28 29 30

Fig. 9. BamH1 digests of mobilized cloning vector pBR328. Lane 1=isolate 1; Lane 2=isolate 2; Lane 3=isolate 3; Lane 4=isolate 4; Lane 5=islolate 5; lane 6=BamH1 digest of Vwa plasmid; Lane 7=BamH1 digest of pBR328; lane 8=pBR328 undigested; Lane 9=isolate 7; Lane 10=isolate 9; Lane 11=isolate 14.


1 2 3 4 5 6 7 8 9 10 11

indicated that mobilization was exclusively the result of Tn1000-mediated transposition (Guyer,1978). Consequently, isolates 4 and 14 were not considered as clones carrying the Vwa origin of transfer. However, this still did not resolve the discrepancy of whether mobilization of pBR328 was a result of the presence of the BamH1-5 fragment or the result of the combination of BamH1-4 and BamH1-9 fragments. In an effort to confirm the presence of Tn 1000 and further clarify the nature of the inserts, isolates 1,2,3, and 4 were double digested with BamH1/Sal1 as well as BamH1/EcoR1 (Fig 10). Comparison of the double digests with the restriction map of Tn 1000 (Fig 11) indicated that isolates 2,3, and 4 contained a 2.5 Kb BamH1/Sal1 fragment as well as a 3.6 Kb BamH1/EcoR1 fragment, consistent with the presence of Tn 1000 in the recombinant molecule but not in double digests of the Vwa plasmid alone. Isolate 1 gave neither fragment and as such the BamH1-5 fragment which lies in the Icr region of the Vwa plasmid resulted in mobilization of pBR328. This plasmid has been designated pVOT-1

To confirm that the BamH1-5 fragment was indeed responsible for mobilization of pBR328, the mobilization of pVOT-1 by F'lac was compared to that of isolate 4 carrying Tn 1000 as well as that of pBR328 alone (Table 7). Mobilization of pYOT-1 was slightly higher than pBR328 carrying Tn1000. More importantly, plasmid pYOT-1 gave almost a tenfold increase in mobilization of the chloramphenicol and ampicillin markers when compared to pBR328 alone. For these reasons I have assigned mobilization of the Vwa plasmid to the BamH1-5 fragment.

Fig. 10. BamH1/EcoR1 and BamH1/Sal1 double digests of mobilized cloning vector pBR 328. Lanes1-6= BamH1/EcoR1 double digests. Lane 1=isolate 1; Lane 2=isolate 2; Lane 3=isolate 3; Lane 4=isolate 4; Lane 5=Vwa plasmid; Lane 6=pBR 328. Lanes 7-12= BamH1/Sal1 double digests. Lane 7=isolate 1; Lane 8=isolate 2; Lane 9=isolate 3; Lane 10=isolate 4; Lane 11=Vwa plasmid; Lane 12=pBR 328. Lane 13=BamH1 digest of Vwa. Lane 14=pBR 328 undigested.

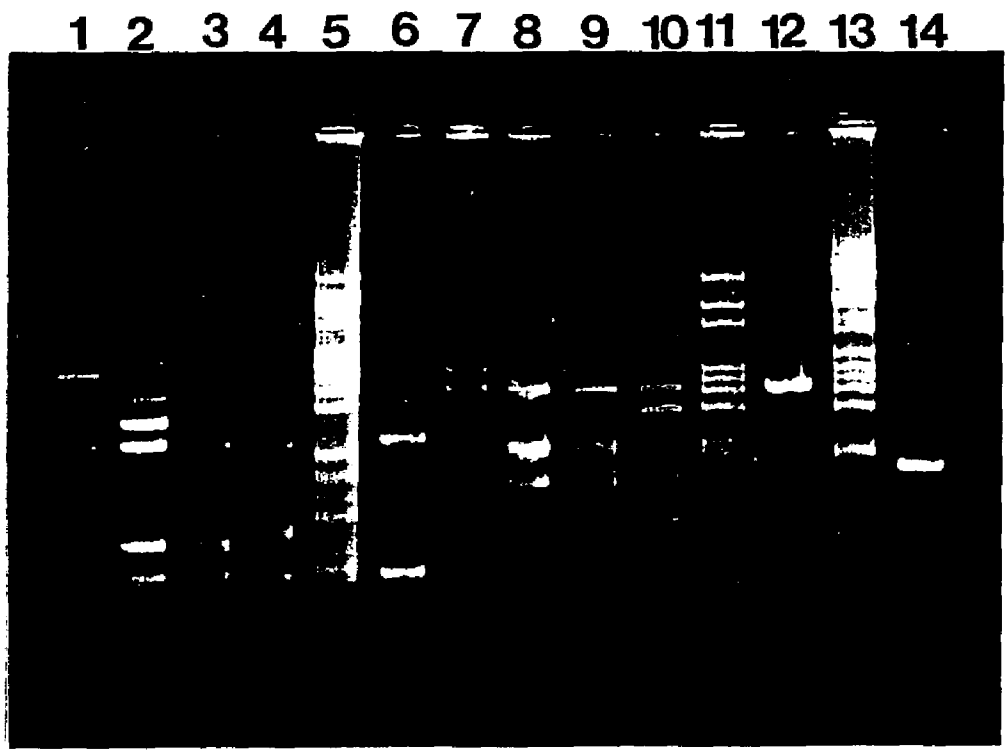

1 2 3 4 5 6 7 8 9 10 11 12 13 14

Fig 11. Restriction map of Tn 1000 found in the fertility factor F (Guyer, 1978). The transposon spans the region from 2.8 to 8.5 Kb in F

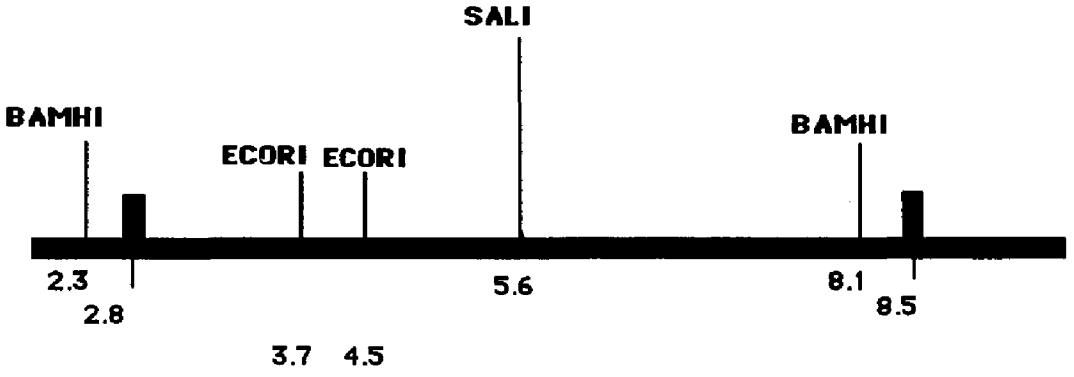

SALI
BAMHI
ECORI
ECORI
BAMHI
2.3
2.8
3.7
4.5
5.6
8.1
8.5

Table 10

% Conjugal Transfer of pBR 328, pBR 328::Tn1000, and pYOT-1

Mating	Selection	%Transconjugants[a]	%Transconjugants[b]
E.coli WIA2 x W1177 (F'lac/pYOT-1)	VRB,Sm	35.0	100
	LB,Sm,Cm,Ap	1.6×10^{-2}	4.6×10^{-2}
E.coli WIA2 x W1177 (F'lac/pBR328::Tn1000)	VRB,Sm	40.0	100
	LB,Sm,Cm,Ap	1.6×10^{-2}	4.0×10^{-2}
E.coli WIA2 x W1177 (F'lac/pBR328)	VRB,Sm	32.0	100
	LB,Sm,Cm,Ap	1.8×10^{-3}	5.6×10^{-3}

a = expressed as percent of donor transfer
b = expressed as percent of F transfer

Phylogenetic Study

The mobilization of the Vwa plasmid and the subsequent cloning of the fragment responsible for this mobilization led me to the consideration of whether or not the Vwa plasmid was a conjugative plasmid in its own right. To determine if the Vwa plasmid shared significant sequence homology with other conjugative plasmids, a survey was conducted with representative conjugative plasmids of various incompatibility groups listed in Table 2.

Figure 12A shows the plasmid profiles of several conjugative plasmids. Figure 12B represents the same plasmids probed with the Vwa plasmid. From this experiment it was evident that the Vwa plasmid showed sequence homology to the Inc P plasmid RP4. To better define the nature of this homology the plasmid RP4 was digested with the restriction endonuclease Sma1 and probed again with the Vwa plasmid (Fig. 13A,B). Sma1 cuts RP4 five times and results in the tra genes being located on different fragments (Fig. 14). If the sequence homology between Vwa and RP4 was due to similarities in the transfer genes, we would have expected the Sma1 fragments 1 and 2 to exhibit hybridization. However, Figure 13B shows that the homology between RP4 and Vwa is located in the Sma1-4 fragment of RP4 (an ill- defined region) and the homology was not due to similarities in the known transfer genes.

Several other plasmids including RA1, R64, R27, pHH1508a, R446b, R402, RA3, Tp231, Sa, and pGC1 were probed with the Vwa plasmid and none showed any degree of sequence homology (data not shown).

Figure 12A. Plasmid profiles of several conjugative plasmids. Lane 1=R621a(Inc I_{gamma}), Lane 2=R16(Inc B), Lane 3=R805a(Inc I,B), Lane 4=N3(Inc N), Lane 5=R387(Inc K), Lane 6=RP4(Inc P), Lane 7=plasmids from Y.pestis EV7651F containing the E.coli plasmid RSF 1030. B. Southern hybridization of gel in (A) using the Vwa plasmid of Y.pestis 019 as probe.

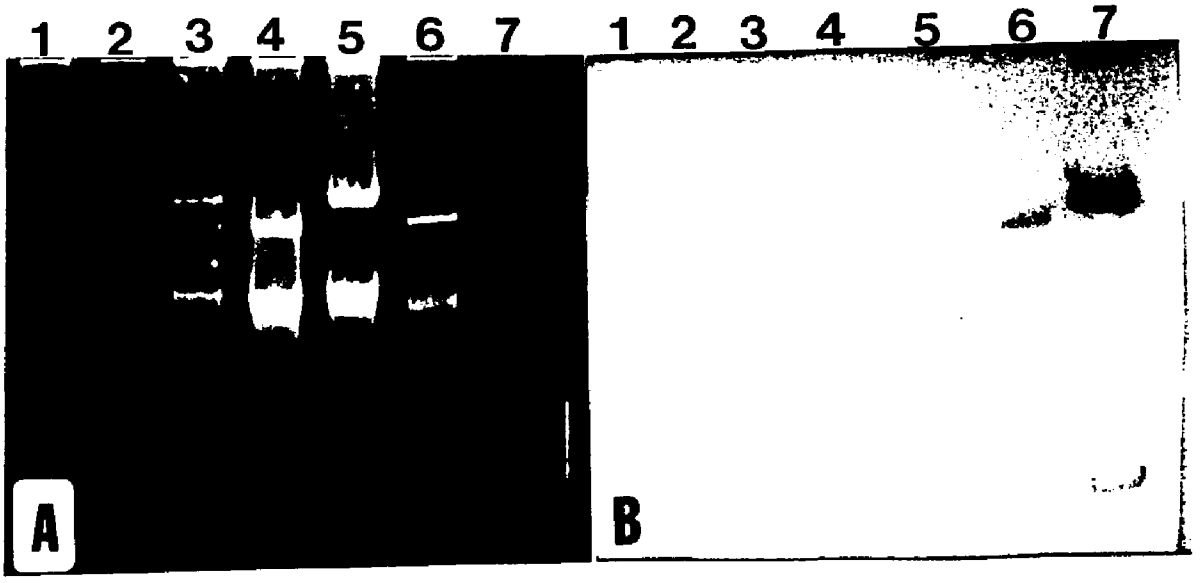

1 2 3 4 5 6 7
1 2 3 4 5 6 7
A
B

Figure 13A. Digest of plasmid RP4. Lane 1=Sma1 digest of RP4 plasmid, Lane 2=BamH1 digest of Vwa plasmid. B. Southern hybridization of gel in (A) using the Vwa plasmid from Y.pestis 019 as probe.

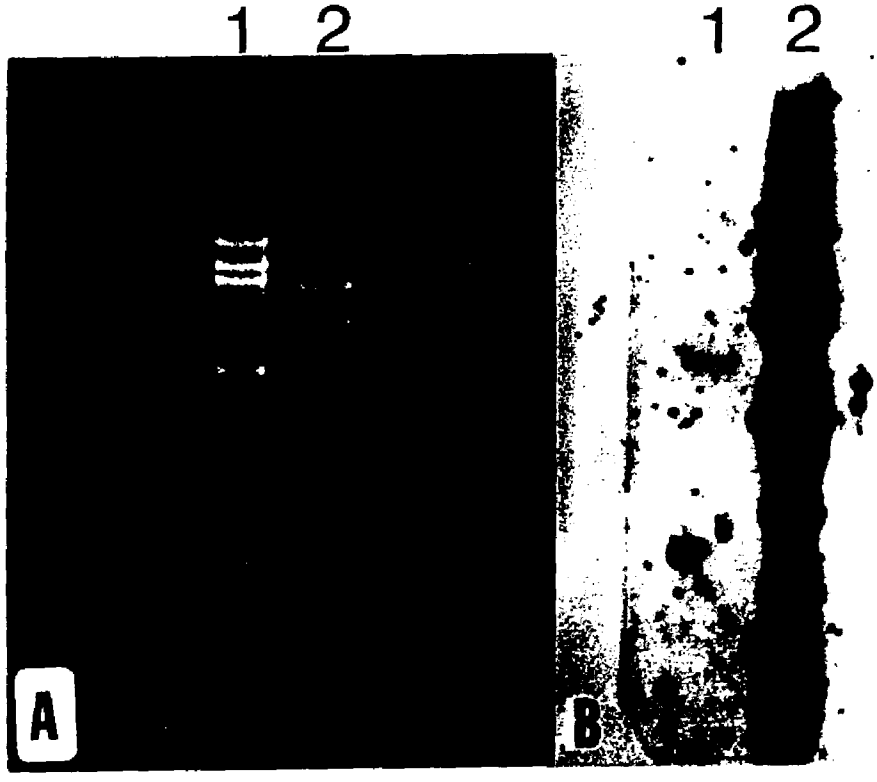
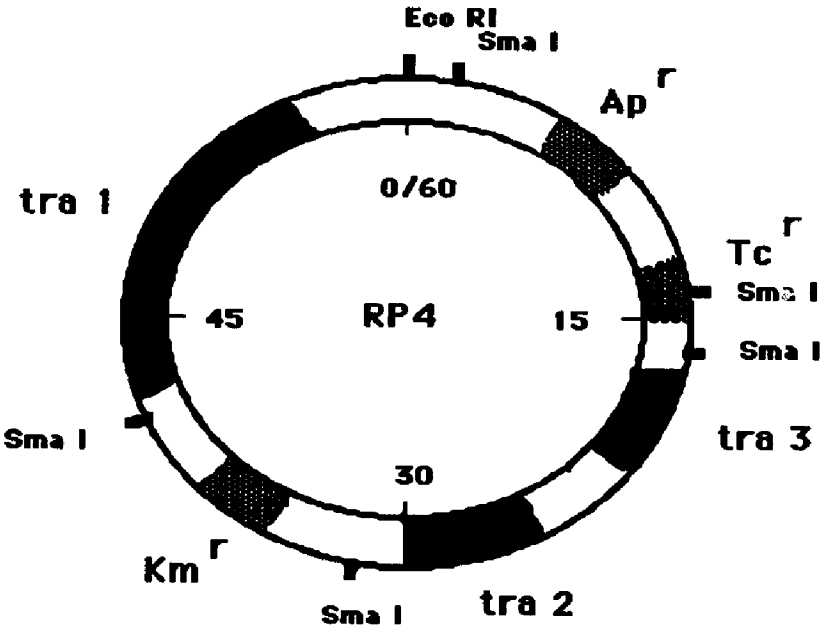

1 2
1 2
A
B

Fig. 14. Restriction map of plasmid RP4 (Lanka et al, 1983)


Eco RI
Sma I
Ap r
tra 1
0/60
Tc r
Sma I
45
RP4
15
Sma I
Sma I
tra 3
30
Km r
Sma I
tra 2

IV. Discussion

Plasmid transfer between Escherichia coli and Y. pestis strains has been reported (Martin and Jacob 1962, Ginoza and Matney 1963, Molnar and Lawton 1971), and recently it was determined that transfer of the fertility factor (F) of E. coli to virulent Y. pestis strains resulted in the loss of virulence (Zsigray et al, 1983). Avirulence was attributed to the loss of calcium dependence, and the plasmid profiles in combination with Southern blots of Y. pestis strains harboring F, showed that the 47 Mdal plasmid had integrated into the Y. pestis chromosome in those transconjugants (Zsigray et al, 1985). A plausable explanation for this observation was plasmid incompatibility, particularly since Bakour et al (1983) have shown that the incD region of F shares sequence homology with a portion of the Vwa pasmid of Y.enterocolitica and Y. pseudotuberculosis.

Two reports have described the mobilization of plasmids from Yersinia. One study dealt with the mobilization of the 6 Mdal plasmid by an R factor (Kol'tsova et al, 1971), and the second study reported mobilization of the Vwa plasmid of Y. enterocolitica. In this study, mobilization apparently required cointegrate formation via recombination between homologous regions on the Vwa plasmid and a 2.9 Kb Bam H1 fragment of pO:8 inserted into a mobilzable vector (Hessemen and Laufs, 1983).

We have shown that the Vwa plasmid of Y. pestis and the F plasmid of E. coli coexisted as autonomous genetic elements in selected E. coli cells. We have further shown that the Vwa plasmid of Y. pestis was mobilized in E. coli strains harboring F. Three mechanisms were considered that could

possibly account for the mobilization of the Vwa plasmid. These included recombinant cointegrate formation, transposon-mediated cointegrate formation, and finally mobilization of the Vwa plasmid via an origin of transfer (ori-T). Mobilization of the Vwa plasmid was independent of recA function, and excluded the first possibility. Likewise, transposon-mediated cointegrate formation would not account for the high frequency of transfer of the pYV019::Tn5 plasmid observed in our studies. Given a donor population of 10^8 cells and a transposition frequency of 1 in 10^{-5}, 1000 transpositions would be expected. Since the F plasmid accounts for approximately 2.5% of the total DNA available for insertion, a total of 25 transpositions into the F plasmid would be predicted. Assuming 30% of the F plasmid was involved in conjugation, this would allow approximately 18 transpositions within the donor population as a result of transposon-mediated cointegrate formation and cotransfer. This corresponds to 1.8×10^{-5}% of the donor population becoming transconjugants. However, this is based on 100% frequency of F transfer. A more realistic transfer rate of 10% would result in a transconjugant frequency of 1.8×10^{-6}. The frequencies we obtained were 100-1000 times greater than can be accounted for by conjugation mediated by the Tn5 and Tn9 transposons. A second consideration that suggested against transposon-mediated cointegrate formation was the lack of a change in molecular weight in the plasmid profiles of the transconjugants. One would envision cointegrate formation as an intermediate in transposition and a change (increase) in molecular size of the plasmid would be expected following resolution of the cointegrate.

The concept that the Vwa plasmid of Y. pestis contains it's own origin of

transfer was drawn from a comparison of Table 11 (summary of tables 3,5,6, and 8) with those frequencies of Table 12. Willetts and Wilkins, (1984) calculated the frequency of transfer for several recombinant plasmids containing various origins of transfer in the presence of different conjugative plasmids. The frequencies they calculated correlated closely to those reported here for the mobilization of the Vwa plasmid of Y. pestis. The results presented strongly suggest that the Vwa plasmid of Y. pestis contains its own origin of transfer capable of mobilization in the presence of a conjugative plasmid (Allen et al, 1987).

In an attempt to clone this origin of transfer of the Vwa plasmid I took advantage of a pBR322 derivative known as pBR328 in which the nic site as well as the mob genes had been deleted. These deletions rendered the cloning vehicle incapable of mobilization. Since ori T occupies a specific site on the plasmid, and plasmids containing such a sequence can be selected via their efficient transfer from bacteria carrying a compatible conjugative plasmid, this cloning vehicle was ideal for the isolation and analysis of DNA fragments carrying an ori T site.

The conjugal transfer of small nonconjugative plasmids through the co-residence of a conjugative plasmid in the donor cell was thought to occur by one of two general mechanisms, conduction or donation (Clark and Warren, 1979). Conduction is a low frequency event which involves the physical association of the two plasmids whereby the small plasmid is transferred with the large conjugative replicon as a consequence of some recombination event involving either recA dependent generalized homologous recombination or transposition. Transposon mediated

TABLE 11: FREQUENCY OF MOBILIZATION OF THE Vwa PLASMID OF Y. PESTIS BY F-CONTAINING STRAINS OF E. COLI

			SELECTIVE MARKERS						
	DONOR	RECIPIENT	Cm	Kn	Nal	Sm	Lac	%Transconjugants[a]	%Transconjugants[b]
1.	K57(F'Cm/pYV019::Tn5)	W1A2	+	-	+	-	-	20.2	100
			-	+	+	-	-	1.8×10^{-4}	8.7×10^{-4}
			+	+	+	-	-	2.6×10^{-4}	1.3×10^{-3}
2.	W1A2(F'Cm/pYV019::Tn5)	W1177	+	-	-	+	-	.21[c]	100
			-	+	-	+	-	2.3×10^{-4}	1.1×10^{-1}
			+	+	-	+	-	1.2×10^{-4}	5.6×10^{-2}
3.	SE5000(F'Cm/pYV 019::Tn5)	W1A2	+	-	+	-	-	13.3	100
			-	+	+	-	-	1.2×10^{-3}	9.1×10^{-3}
			+	+	+	-	-	1.3×10^{-2}	9.8×10^{-2}
4.	HfrC(pYV019::Tn5)	W1A2	-	-	+	-	+	1.29	100
			-	+	+	-	-	4.1×10^{-5}	3.2×10^{-3}

a = expressed as percent of donor input
b = expressed as percent of F transfer
c = mating terminated after 10 minutes

Table 12

Frequencies of transfer of different origins of transfer with various conjugative plasmids[1]

Conjugative Plasmid	Mobilization[2]		
	pED822 (oriT-F)	PED221 (oriT-R1)	pED222 (oriT-R100)
F'lac	140	6×10^{-4}	2×10^{-3}
pED219	2×10^{-3}	112	4×10^{-3}
R100drd-1	0.3	7×10^{-3}	205

[1] Reprinted with permission from the American Society for Microbiology

[2] Expressed as percentage of the transfer of the conjugative plasmid

conduction is generally assumed to be less specific, resulting from transposition by transposons on either the conjugative or nonconjugative plasmids. The presence of Tn 1000 in many of the mobilized pBR328 clones was consistant with an earlier report in which mobilization of pBR322 by an F plasmid was primarily through random insertion into pBr322 (Guyer, 1978). Also consistant with this report was my finding that none of the clones carried IS2 or IS3 from F. It should be noted here that there is evidence for another low-frequency mechanism for conduction of nonconjugative plasmids by F (Goto et al, 1984).

Donation involved no physical association between the two plasmids but did require certain genes on the non conjugative replicon: usually those associated with trans-acting proteins (the mobility proteins) and a cis-acting site. It was this cis-acting site which is thought to be the origin of transfer. The mobilization of pBR328 carrying the BamH1-5 from the Vwa plasmid suggested that these sequences contained the ori T. The possibility of generalized homologous recombination was eliminated by virtue of using a recA donor, as well as the fact that an earlier study showed no sequence homology between F and the Icr region of Vwa (Bakour et al, 1983). In addition, the specificity of the mob gene products of F (Willetts and Wilkins, 1984) argues against the possibility of a second DNA sequence in the Vwa plasmid other than the ori T which was recognized by F. It was for these reasons that I have assigned the ori T of the Vwa plasmid of Y. pestis to the BamH1-5 fragment.

My phylogenetic study revealed that the Vwa plasmid of Y. pestis showed sequence homology with the Sma1-4 fragment of plasmid RP4. This was a very poorly defined region of RP4 which was somehow involved

in stable replicon maintenance (Thomas, 1981). An earlier study has shown that the Vwa plasmid shares sequence homology with the inc D region of F (Bakour et al, 1983). With these two exceptions our hybridization studies did not reveal any significant homology between the Vwa plasmid and representative conjugative plasmids from virtually every incompatibility group among the Enterobacteriaceae. From these data we draw one of three conclusions: 1) the Vwa plasmid is not a conjugative plasmid; 2) that the Vwa plasmid shares sequence homology to a conjugative plasmid outside of the Enterobacteriaceae; 3) the Vwa plasmid is a conjugative plasmid of an undefined incompatibility group. Thus, additional studies are necessary to determine the phylogenetic nature of the Vwa plasmid harbored by the Yersinia.

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